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The Role of Stem Cells in Fibrous Dysplasia of Bone and the Mccune-Albright Syndrome

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Abstract

Stem cells have become a major area of interest in the treatment of human disease, but more recently, stem cells have come to be appreciated as the cause of disease. Fibrous dysplasia of bone and the McCune-Albright Syndrome evolve from activating missense mutations in $G_s\alpha$ in pluripotent embryonic stem cells. The legacy of these mutations remains in a population of mutated multipotent post-natal skeletal stem cells ("mesenchymal" stem cells), which direct the formation of abnormal bone and a fibrotic marrow in fibrous dysplasia. Future therapeutic approaches for the treatment of fibrous dysplasia, the most significant component of the McCune-Albright Syndrome, will depend on a greater understanding of post-natal skeletal stem cell biology and how skeletal stem cells can be manipulated for efficient bone regeneration.

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Key words: Mutations; Somatic Mosaic; $G_s\alpha$; Embryonic Development

Introduction

In the past decade, "stem cells" have become a major topic of interest, not only for the scientific community, but also for the population at large. The remarkable ability of stem cells of embryonic, fetal or post-natal origin to reform tissues, puts them at the fore front of a biologist's desire to understand the mechanisms by which tissues are formed and how they are maintained. But to the lay population, "stem cells" have come to represent an everlasting cure for diseases and disorders that to date can only be treated by palliative means.

Although the full definition of a stem cell within a given post-natal tissue is a moving target, there are a number of parameters that all can agree upon based upon what is known about the activity of stem cells within the human body (see http://stemcells.nih.gov/info/basics/basics2.asp). First, a single stem cell is able to give rise to progeny that form all of the cell types within a given tissue. Second, a stem cell is able to maintain itself by self-renewal. Self-renewal of stem cells in humans is not well understood to date, but could occur via several different mechanisms (1). A stem cell could divide asymmetrically, with one daughter cell remaining a stem cell and the other becoming a more differentiated cell that transiently amplifies and subsequently differentiates. In another scenario, a stem cell could divide with both daughters remaining as stem cells, or both daughters becoming more differentiated transiently amplifying cells (symmetric division). In this view, self-renewal would be a random event, or dictated by the local circumstances in which a stem cell resides. Lastly, it cannot be ignored that in some situations, what would be defined as a fully differentiated, post-mitotic cell may become proliferative and reform cell types within a given tissue (2). A current read of the literature would ascribe to the existence of post-natal stem cells in virtually every tissue in the body, based on the ability to establish populations of cells with "stem cell-like" properties in culture, coerced to undergo differentiation into multiple phenotypes by in

vitro differentiation assays (3). However, the gold standard by which the differentiation capacity of a stem cell can be assessed is by in vivo transplantation assays and demonstration of cell function. To date, this type of rigorous analysis is lacking in many cases. Nonetheless, stem cell biology has provided a new perspective not only on the mechanisms of tissue turnover, but also in gaining a better understanding of the pathogenetic mechanisms of disease and their possible usage in the treatment of disease. What follows below is a description of how FD/MAS evolves as a disease of stem cells, during both embryonic development and post-natal growth and future prospects for the use of stem cells in the development of novel therapies.

FD/MAS

Fibrous dysplasia of bone (FD) can occur both outside of and within, the context of the McCune-Albright Syndrome (MAS, OMIM 174800), both of which are genetic, but not inherited diseases. As described else where in this volume (see Zacharin, Chapter 6), the three phenotypic manifestations of MAS include hyperpigmented skin lesions (café au lait spots with the "coast of Maine" contour), FD and hyperfunctioning endocrinopathies including precocious puberty, hyperthyroidism, growth hormone excess and Cushing's disease. In addition, other tissues such as the liver, pancreas, cardiac and skeletal muscle may be involved [reviewed in (4,5)].

It has been demonstrated that all three phenotypes (café au lait, FD and hyperfunctioning endrinopathies) are caused by the same missense mutations that occur post-zygotically in the GNAS complex locus that encodes for the activating G protein, $G_s\alpha$, on chromosome 20 (6-8). Two mutations have been shown to be the most common cause of all three phenotypes: replacement of arginine at codon 201 with cysteine or histidine (R201C, R201H). The mutations result in a loss of the intrinsic GTPase activity of $G_s\alpha$, which leads to constitutive activation of adenylyl cyclase and over production of cAMP (9). The impact of these mutations in the formation of café au lait hyperpigmentation and hyperfunctioning endocrinopathies is immediately apparent due to the known association of $G_s\alpha$ with various G protein coupled receptors in these tissues, whereas the impact on skeletal tissues is only more recently coming to light (see Bianco and Riminucci, Chapter 5) (4,5).

Skeletal involvement can be restricted to a single bone (monostotic), multiple bones (polyostotic), or virtually every bone in the body (panostotic). Regardless of the extent of the skeletal disease, fibrous dysplastic lesions are characterized by replacement of normal bone and marrow by abnormal bone and a fibrotic marrow devoid of hematopoietic elements (see Bianco and Riminucci, Chapter 5) (10). While patients with monostotic disease often do not have associated café au lait lesions or hyperfunctioning endocrinopathies, the majority of patients with polyostotic disease have either one or both of these distinguishing features. Likewise, individuals can present with isolated café au lait hyperpigmentation, or a single hyperfunctioning endocrinopathy. Consequently, these patients present in a broad range of phenotypic traits and disease severity, the latter due not only to disease burden, but also due to the impact of hyperfunctioning endocrinopathies on fibrous dysplastic bone (11,12). How this diversity is established is rooted in development, the mutation of pluripotent embryonic stem cells and the migration and misfunction of their more differentiated progeny (13).

Mutation of Embryonic Stem Cells as the Cause of FD/MAS

Due to the post-zygotic nature of the activating missense mutations of GNAS, it has been speculated that the phenotypic heterogeneity and disease severity noted between patients with MAS is due to the time and place in which the mutation occurs. That is, that the mutation would have occurred earlier during embryonic development in a patient with widespread

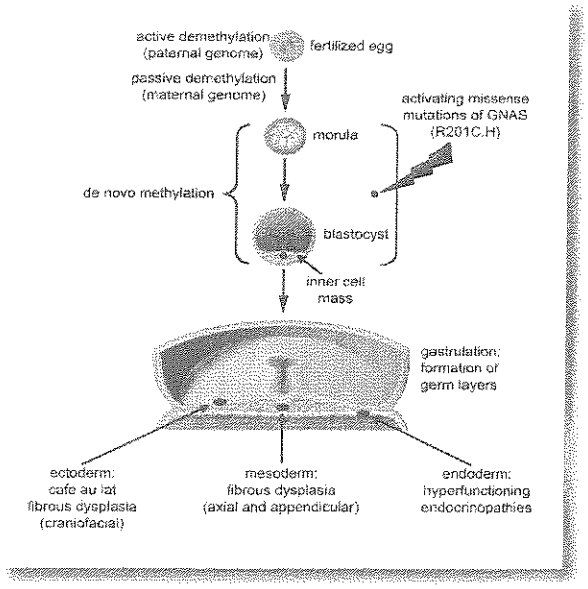

Figure 1. Human Embryonic Development
Following fertilization, there is active demethylation of the paternal genome. Upon subsequent division to form blastomeres, the maternal genome undergoes a slow, passive demethylation. At the 16-32 blastomere stage (a morula) and in the blastocyst, de novo methylation occurs in cells that are rapidly proliferating. It is between these two stages that isolated CpG dinucleotides can become methylated and subsequently deaminated to generate R201C and R201H mutations (see also Figure 2). Cells mutated prior to gastrulation can subsequently migrate and differentiate in ectodermal, mesodermal and endodermal tissues, giving rise to café au lait and craniofacial fibrous dysplasia, axial and appendicular fibrous dysplasia and hyperfunctioning endocrinopathies.

and severe disease, whereas the mutation would have occurred later in patients with more limited disease (8,14). In this view, the extent of disease would be established by when a single cell became mutated and where its progeny migrated. However, there is little experimental evidence to support this notion and current observations suggest a more likely scenario in all cases of patients with MAS.

The tissues and organ systems affected in MAS represent derivatives of all three embryonic germ layers: ectoderm (café au lait, craniofacial FD), mesoderm (axial and appendicular FD, hyperfunctioning endocrinopathies) and endoderm (hyperfunctioning endocrinopathies) (**Figure 1**). The presence of phenotypes that arise in cells from all three germ layers in a single patient indicates that the mutational event must occur before the three embryonic germ layers are formed; that is, before gastrulation. Otherwise, multiple and identical, mutational events would have to occur in different cells in different places, which while not virtually impossible, is highly unlikely.

FD/MAS Mutations Occur prior to Gastrulation

During development, the fertilized egg is a totipotent cell, capable of giving rise to not only embryonic, but also extraembryonic tissues. The fertilized egg divides to form blastomeres and it is currently thought that up until the four cell stage, each blastomere remains totipotent. As division proceeds, the morula is formed (16-32 blastomeres), the outer most of which continue to divide to form a single outer layer of trophoblasts (which mediate invasion of the zygote into the uterine wall), while the inner most cells divide to form the inner cell mass of the next stage of development, the blastocyst (see http://www.visembryo.com/baby/index.html). Within the inner cell mass, hypoblasts will form only the extraembryonic membranes. The remaining cells, epiblasts, are the pluripotent cells that are capable of giving rise to all cell types of the body (and are the source of embryonic stem cells that can be established in vitro). During gastrulation, the three embryonic germ layers are formed (ectoderm, mesoderm and endoderm) and the cells within these layers become irreversibly committed to forming structures of ectodermal, mesodermal and endodermal origin (**Figure 1**) (15). While cells within a specific germ layer are multipotent and capable of forming numerous cell types, tissues and organs, they are incapable of forming those of another germ layer (15). However, it is also notable that two different germ layers give rise to bone (16). The majority of the craniofacial bones derive from neural crest of ectodermal origin (17), while axial and appendicular bones derive from mesoderm.

Based on this pattern of embryonic development, it is apparent that the mutational event must occur prior to gastrulation in order for MAS to be established. However, the implications reach even further. In patients with a craniofacial FD lesion or a femoral lesion (the two most common sites for FD to occur) (18), but lacking other phenotypes, the mutation must have occurred prior to gastrulation due to the dual germ layer origin of craniofacial and appendicular bones. Similarly, the same timing of mutation also applies to patients that present with FD and a hyperfunctioning endocrinopathy or café au lait hyperpigmentation, or to patients with a hyperfunctioning endocrinopathy and café au lait hyperpigmentation. Based on current clinical observations, patients displaying phenotypic abnormalities derived from two or three of the embryonic germ layers represent the vast majority of patients with these mutations. Although polyostotic FD had been originally thought to occur independently of MAS, careful clinical analyses have revealed that many of these patients have occult endocrinopathies, or unnoticed café au lait spots. However, patients with truly monostotic disease, or those with café au lait, most likely represent mutational events occurring very late during embryogenesis and perhaps even post-natally as in the case of acquired endocrinopathies (the so call *gsp*-mutations) (19).

Although it is apparent that the mutational event occurs prior to gastrulation in most patients, what establishes the notable heterogeneity in the localization and the severity of disease in the patient population is less clear. After mutation of a single cell, its progeny will be distributed throughout the body based on developmental clues, which are not fully elucidated, that specify where the cells will migrate and what they will develop into. Establishment of lesions may be related to the survival of the mutated cells. To date, there has been no documentation of a germline transmission of FD or MAS, which has lead to Happle's hypothesis that germline mutations would be embryonic lethal and that the $G_s\alpha$ R201-mutated cells can only survive due to somatic mosaicism (19). Consequently, the establishment of lesions within skin, bone and endocrine tissue may depend on the concentration of mutated cells and their ability to survive. Areas in which too many mutated cells reside may result in their demise and lack of establishment of a lesion. Another possibility that may contribute to variability in disease burden is based on the levels of expression of $G_s\alpha$. While it is often stated that $G_s\alpha$ is ubiquitous, the level of expression may vary depending on the cell type or stage of maturation. For example, mutated cells have been found in the blood of some patients with FD/MAS, yet these patients do not have an overt hematopoietic phenotype, suggesting that $G_s\alpha$ is either not expressed or expressed at insignificant levels in blood cells. It has also been reported that while $G_s\alpha$ is detectable in pre-osteogenic mesenchyme and there is a dramatic upregulation as these cells differentiate into fully mature osteoblasts, which subsequently misfunction to form abnormal bone (see Bianco and Riminucci, Chapter 5) (10). Lastly, the GNAS complex locus that gives rise to multiple

transcripts including $G_s\alpha$ undergoes a complex pattern of imprinting. In some tissues, $G_s\alpha$ is expressed primarily for the paternal allele while in others, it is expressed from the maternal allele, consequently a mutated allele may be silenced in certain tissues (20).

Cytosine Methylation Gives Rise to Both R201C and R201H Mutations

The R201 codon (CGT) in $G_s\alpha$ is a mutational hot spot with R201C (C→T, TGT) and R201H (G→A, CAT) accounting for virtually all of the mutations in FD/MAS (with rare reports of R201S and R201G) (4,5,21,22). The R201 codon includes a CpG dinucleotide in both the sense and anti-sense direction. Isolated CpG dinucleotides rarely occur in the human genome, with the exception of CpG islands, which are frequently methylated in imprinted genes. Methylation of cytosine in isolated CpG nucleotides often occurs, as there is a tendency to eliminate them from the genome. While there are repair mechanisms to remove methyl groups, if they are not removed prior to DNA replication, subsequent deamination of the methylated cytosine results in a C→T transition in the sense strand (R201C) and a G→A transition in the anti-sense strand (R201H) (**Figure 2**). Interestingly, transitions in CpG dinucleotides leading to the substitution of arginine by either cysteine or histidine, comprise ~30% of the mutations known to cause human genetic diseases. Thus, while patients with FD/MAS are relatively rare, their mutations arise by a fairly common mutational mechanism. What has not been well recognized until recently is that in FD/MAS, the two common mutations arise via the same mechanism (methylation and deamination), on the sense and anti-sense strand of the R201 codon. In our patient cohort, the number of patients with the R201C mutation is roughly equivalent to those with the R201H mutation, suggesting that both strands of DNA are equally sensitive to methylation.

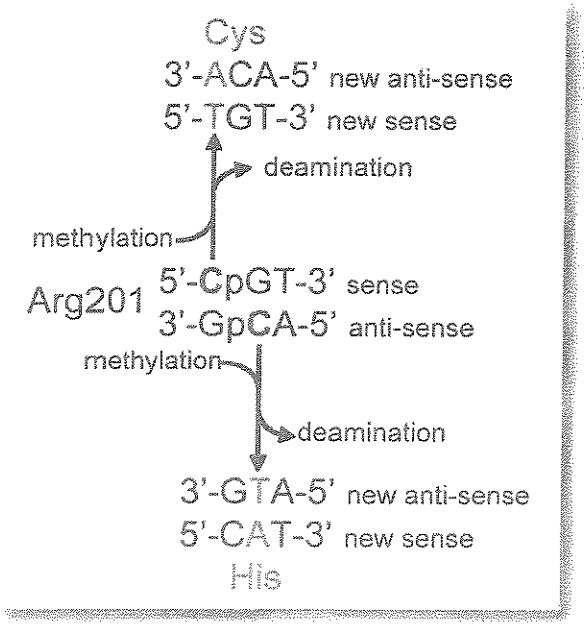

Figure 2. Generation of R201C and R201H Mutations in Gsα
The R201 codon has a CpG dinucleotide, in both the sense (5'-CpGT-3") and ant-isense (3'-ACpG-5") directions. Methylation of the cytosine bases and subsequent demethylation results in a C→T conversion (TGT, R201C) and a G→A (CAT, R201H)

Methylation during Embryonic Development

Methylation of CpG dinucleotides and CpG islands is a phenomenon that plays a central role in epigenetic control, which is critically important during development in determining the pattern of gene expression of cells as they become more committed to one fate or another, without a change in genomic sequence. Furthermore, methylation controls imprinting of genes such that either the maternal or paternal allele is silenced, as is known for many of the alternative GNAS transcripts (23,24). There is increasing awareness that disruption of the normal pattern of epigentic control of gene expression is highly associated with development of disease.

Following fertilization, the paternal genome undergoes rapid demethylation (active demethylation) and upon subsequent divisions, the maternal genome is slowly demethylated (passive demethylation). As a consequence of nuclear localization of DNA methyltransferase 1, de novo methylation occurs as the zygote progresses from the morula through the blastocyst stages of development to establish the pattern of imprinting of certain genes and to control the pattern of gene expression (**Figure 1**) (25). Of note, in the blastocyst, the rate of methylation is much higher in the inner cell mass than in the trophoblastic layer (25) and it is within this milieu that CpG dinucleotides can be accidentally methylated. Under normal circumstances, repair mechanisms would remove the methyl group prior to DNA replication. However, the high rate of proliferation of cells in the morula (the inner most blastomeres of which are destined to become the inner cell mass) and subsequently in the inner cell mass, may increase the chance that it is not, resulting in the base transition and establishment of the mutation, as is reflected by the high occurrence of C→T and G→A mutations in human diseases.

The understanding of this mechanism of mutation during embryonic development, along with the fact that in FD/MAS, mutated cells are derived from all three germ layers lends further support to the notion that the mutational event must occur prior to gastrulation and most likely occurs between the formation of the morula and the blastocyst, in a pluripotent cell that will ultimately become an epiblast in the inner cell

mass. Thus, first and foremost, FD/MAS is a disease established by an embryonic stem cell.

FD as a Post-Natal Skeletal Stem Cell Disease

FD evolves in the post-natal individual as a disease of the bone/marrow organ as a whole, establishing it as a disease not only of osteoblasts, but also as a disease of the lineage to which osteoblasts belong. FD lesions develop as both bone and marrow are replaced with structurally unorganized and unsound bone and a fibrotic tissue that is devoid of hematopoiesis and marrow adipocytes (4,10). This abnormal pattern is rooted in the shared origin of osteoblasts, myelosupportive stroma and adipocytes, not only during embryonic development, but also in their re-derivation from a common stem cell during post-natal growth, bone turnover that occurs throughout life and in repair of damaged bone tissue (26).

Skeletal Stem Cells

It is now well recognized that at least two populations of stem cells reside in the post-natal bone marrow: the hematopoietic stem cell and the skeletal stem cell (also known as the bone marrow stromal stem cell and the "mesenchymal" stem cell). By taking single cell suspensions of bone marrow and plating them at low density, a small population of bone marrow stromal cells (BMSCs) becomes rapidly adherent and in human specimens, they can be easily separated from non-adherent hematopoietic cells. These specialized BMSCs are capable of density-independent growth and proliferate to form colonies, each one initiated by a Colony Forming Unit-Fibroblast (CFU-F) (27,28). Analysis of individual colonies indicates that approximately 10-20% are able to reform bone, myelosupportive stroma and marrow adipocytes upon transplantation in vivo in conjunction with an appropriate scaffold (29). When cultured in pellet cultures in vitro, they form cartilage. By virtue of the fact that they are able to reform all cell types found in skeletal tissue, they qualify as bona fide post-natal skeletal stem cells (30), although evidence of self-renewal is circumstantial to date, as is required for a true stem cell.

The subset of skeletal stem cells present in the BMSC population play a critical role in post-natal skeletal homeostasis, not only because of their participation in new bone formation, but also due to the fact that they support hematopoiesis and control the differentiation and activity of osteoclasts. Consequently, any deleterious alteration in the biological activity of a skeletal stem cell, caused by either an intrinsic change in its character (mutation), or an extrinsic change (changes in the skeletal stem cell's microenvironment) is likely to lead to a skeletal disorder. In this regard, the skeletal stem cell can be considered to be the unit of skeletal disease (31).

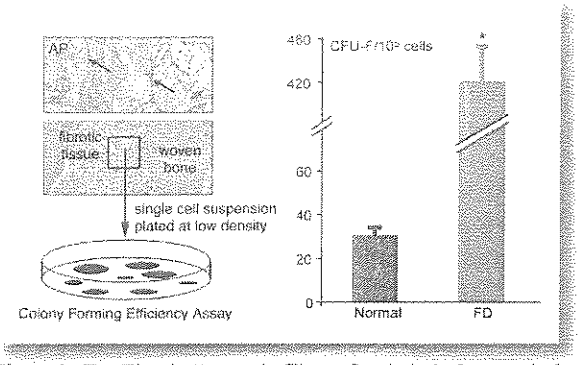

Figure 3. The Fibrotic Marrow in Fibrous Dysplasia is Composed of Misfunctioning Bone Marrow Stromal Cells

The fibrotic marrow in fibrous dysplasia consists of an accumulation of misfunctioning bone marrow stromal cells, characterized by expression of alkaline phosphatase (AP). When single cell suspensions of the fibrotic marrow of FD lesions are explanted at low density, there is a notable increase in the colony forming efficiency of the bone marrow stromal cell population, a subset of which are multipotent skeletal stem cells.

The Fibrotic Marrow in FD is Composed of Misfunctioning BMSCs and Skeletal Stem Cells

At first glance the fibrotic marrow in FD lesions would appear to be a non-descript accumulation of fibroblasts. However, it is notably different in that these cells share many characteristics of bone marrow stromal cells, in particular, the expression of alkaline phosphatase, a marker that is not expressed by fibroblasts of other connective tissues. Histochemical staining for alkaline phosphatase reveals a massive accumulation of BMSCs in the fibrotic marrow (**Figure 3**) (10). This accumulation of BMSCs is further appreciated by performing a colony forming efficiency assay to enumerate the number of CFU-Fs. When a single cell suspension of the fibrotic marrow is prepared by mechanical disruption and plated at low density, the number of CFU-Fs is dramatically increased compared to normal bone marrow (**Figure 3**), confirming the histological observation of an expanded stromal cell population.

Based on the fact that in normal bone marrow, the BMSC population contains a subset of skeletal stem cells that are able to reform a bone marrow organ upon in vivo transplantation, it would stand to reason that FD BMSCs would presumably contain a subset of mutated skeletal stem cells that upon in vivo transplantation would regenerate an FD-like ossicle. When non-clonal populations of FD BMSCs were transplanted in conjunction with hydroxyapatite/tricalcium phosphate ceramic particles, only woven bone was formed by misfunctioning osteoblasts, along with a fibrous tissue that lacked marrow elements (**Figure 4A**). The transplanted cells formed a virtual replica of a native lesion, providing proof of principle of the

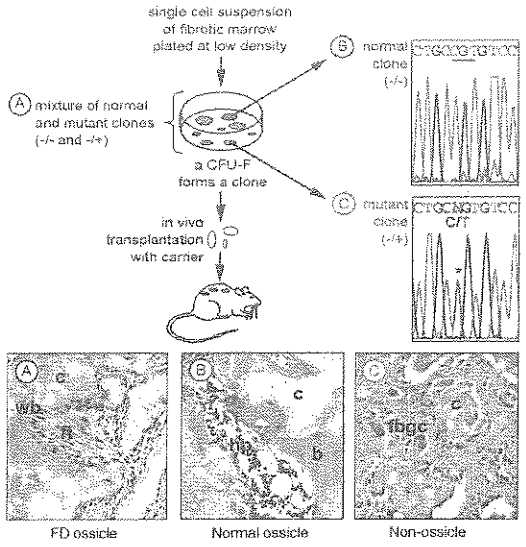

Figure 4. Fibrous Dysplastic Lesions are Mosaics of Normal and Mutant Cells
Upon ex vivo expansion, non-clonal strains derived from FD lesions gave rise to a replica of FD tissue (FD ossicle), with woven bone (wb) and fibrous tissue (ft) upon in vivo transplantation with a carrier (c) (A). When individual clones were established in culture, it was found that a number of clones had a normal genotype and generated normal bone (b) and supported bone marrow formation (hp) (Normal Ossicle) (B). Other clonal lines were of mutated genotype and upon in vivo transplantation, they did not survive (Non-ossicle). These transplants were populated by murine foreign body giant cells (fbgc) (C).

skeletal stem cell within the BMSC population as the causative element in skeletal disease (32).

In analyzing the population of FD BMSCs further, it was noted that the level of mutation that could be detected by a variety of different techniques (direct DNA sequencing, PNA clamping which blocks amplification from the normal allele and a PNA-FRET technique) was highly variable from one sample to another (32-34). Subsequently, clones of individual FD CFU-Fs were isolated and upon genotyping, it was discovered that the FD BMSC population is a mixture of normal and mutated cells. When transplanted in vivo, clones with a normal genotype formed a normal ossicle, with abundant lamellar bone and a hematopoietic marrow, as would be expected (**Figure 4B**). Surprisingly, when clones with a mutant genotype were transplanted, the human mutated cells did not survive and formed a "non-ossicle;" that is, the transplants were populated by foreign body giant cells of mouse origin (**Figure 4C**) (32). These data indicate that not only are the patients somatic mosaics, but that the lesions are mosaics as well. The inability of 100% mutant cells to survive further supports Happle's hypothesis concerning the lethal nature of the mutation and that mutant cells can only survive when supported by normal cells (32).

From these data, it is also apparent that the ratio of normal to mutant cells can have an impact on the nature of FD lesions. While it is not known what level of mutational load is required to form a lesion, it is known that the phenotypic character varies not only from lesion to lesion in the same patient, but also within different areas of a lesion. This variability relates to the morphology of the misfunctioning osteoblasts, with some areas displaying a more retracted appearance and formation of Sharpey's fibers than others (10,35). In some lesions, there are areas in which there is an abundance of osteoclasts, not only on the abnormal bone surface, but also in association with retracted cells in the fibrotic tissue. Over-exuberant osteoclastogenesis is based at least in part on the constitutively high levels of IL-6 expression by mutant cells compared to normal cells (36). Lesional variations can also be seen at the level of bone scans, where some lesions are very active, while others are not, even though they are radiographically abnormal (12). Further work is needed to more precisely determine the impact of mutational load within lesions and if and how load can be modified in a beneficial way.

In addition to differences in the ratio of mutated to normal cells, asymmetric allelic expression from the paternal or maternal allele may also contribute to lesional variation. As mentioned above, many of the alternative transcripts of the GNAS locus, including $G_s\alpha$, are imprinted in different tissues. We have recently demonstrated that when non-clonal normal and FD BMSCs are analyzed, transcription appears to be equal from both the paternal and the maternal allele. However, in individual normal and FD-mutated clonal strains, each arising from a single CFU-F, the ratio of $G_s\alpha$ allelic expression was randomly established within the clones, with a range of 20-80% preference for one allele versus the other (37). This result suggests that a parental-independent modulation of $G_s\alpha$ expression occurs in CFU-Fs. Consequently, the presence of the mutation on the predominantly transcribed allele may impact on the severity within focal areas of an FD lesion.

The Utility of Stem Cells in FD Research

With the discovery of the causative mutation in FD/MAS, the use of cell cultures derived from FD tissue has provided a great deal of information on the derangement of stromal and osteoblastic metabolism as a result of constitutive activity of $G_s\alpha$ that could not be surmised based solely on clinical

observations, or even with histological approaches. The most notable of these findings is the mosaic nature of the lesion itself and the lethal nature of the mutation (31). Furthermore, the discovery that mutated stem cells in combination with normal stem cells within the bone marrow stromal cell population are capable of recapitulating lesional bone upon in vivo transplantation has provided the first small animal model of this disease (31), which can be utilized to study the role of mutational burden on lesion development, the life history of the mutated cells with aging of the lesion and in the future, for testing of new therapeutic approaches.

While much has been learned from these cell populations, there are several disadvantages in the use of primary and subsequent cultures. First, due to the rarity of the disease, fresh lesional tissue is not always available for establishment of new cultures and there are limited supplies of cells for carrying out further studies. Second, the mutational load, which varies from lesion to lesion and from patient to patient, introduces a layer of complexity in interpretation of results. It is for this reason that recent studies have employed the use of single colony derived strains of both normal and mutant cells in order to better reveal the down stream effects of the mutation on cellular metabolism (36). However, it is this aspect that raises the third point, which is that cloning is not only labor intensive, but it is difficult to generate sufficient numbers of cells from a single CFU-F before it becomes senescent.

For these reasons, the use of viral vectors bearing the mutation that stably integrate into the genome and transfer the diseased phenotype provide an attractive alternative. By transducing normal BMSCs (and their complement of skeletal stem cells) with such vectors, more consistent populations of cells could be better utilized to determine the level of mutational load necessary to generate a lesion and to study changes in cell metabolism by genomic and proteomic analyses. Notably, it has been remarkably difficult to either transiently or stably transduce human BMSC populations with sufficient efficacy using a broad variety of non-viral and viral vectors methodologies. However, recent studies indicate that lentiviral vectors, encoding for either marker proteins such as eGFP and even for mutated forms of $G_s\alpha$, can be used to effectively transduce human BMSCs, thereby providing useful reagents for future studies (38). In addition to the use of genetically modified human BMSCs, molecular engineering of murine ES cells and their subsequent introduction into murine blastocysts for the generation of transgenic mice provides a unique avenue by which to study the impact of $G_s\alpha$ during development. In this way, the migration and subsequent differentiation of a murine embryonic stem cell can be systematically studied in a way that would not be possible in the human condition.

Stem Cells for the Treatment of FD

The major sequelae of $G_s\alpha$ mutations that cause morbidity and mortality in patients with FD/MAS are the hyperfunctioning endocrinopathies and fibrous dysplasia of bone. Hyperfunctioning endocrinopathies can be treated, in most cases, by either pharmacological management, or by surgical removal of misfunctioning endocrine tissue and subsequent hormone replacement therapy. What is far less manageable is fibrous dysplasia. Currently there is no "cure" for FD, as there is no cure for any genetic disease of bone. While bisphosphonate treatment has been envisioned as a way of preventing growth of FD lesions, a recent study indicated that bisphosphonates did not prevent FD lesion growth (39). Surgical intervention, while necessary in many instances to provide stability and prevent deformity, is only palliative and in many cases, is unsuccessful due to the lack of solid, normal bone in which to anchor orthopaedic devices (5). There is a

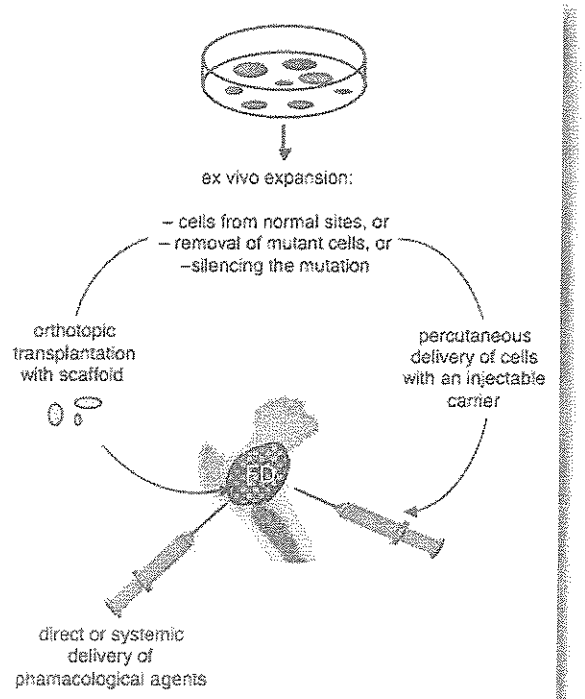

Figure 5. Stem Cells in the Treatment of Fibrous Dysplasia of Bone. Fibrous dysplastic lesions could be treated by the isolation of BMSCs from sites unaffected by disease. If such sites are not available, it may be possible to eliminate mutant cells by FACS, or to silence them via molecular engineering. Once an appropriate population is obtained, direct orthotopic transplantation along with an appropriate scaffold, or percutaneous injection may be utilized to bring about new bone formation within an FD lesion. Alternatively, pharmacological agents, including viral vectors, could be injected either directly into the lesion, or systemically, in order to silence the mutation, or to eliminate the mutated cells.

real need to develop new and more effective methods for the treatment of FD. Towards this aim, harnessing the power of post-natal skeletal stem cells to regenerate new structurally sound bone within FD lesions, comes to the forefront of potential new therapies. There are a number of ways that stem cells could be utilized in the treatment of FD **(Figure 5)** (40,41).

Given the fact that many patients with FD/MAS have unaffected bones, bone marrow from these bones could be isolated and expanded ex vivo to generate a population of BMSCs. These cells could then be used to reconstruct an FD lesion through open surgery and transplantation of the cells along with an appropriate scaffold, such as hydroxyapatite/tricalcium phosphate, which is well known to support the formation of bone when transplanted along with BMSCs. As an alternative to open surgery, it can be envisioned that following aspiration of the contents of an FD lesion, normal BMSCs could be combined with an injectable carrier to hold them in place and conduce their subsequent differentiation and introduced via a percutaneous injection.

However, there are patients that do not have a reasonably accessible source of normal bone marrow and alternative methods must be utilized to obtain a population of normal BMSCs for either orthotopic or percutaneous delivery of cells. In cases where only a mixed population of normal and mutant cells can be obtained, cloning of individual CFU-Fs and their progeny and subsequent genotyping, could be used to generate a population of 100% normal BMSC strains. Far better would be the use of FACS in order to separate normal BMSCs from mutant BMSCs, thereby eliminating the need for cloning. This will require the identification of cell surface markers that effectively distinguish between normal and mutant BMSCs. In cases where the majority of cells are mutant, the use of siRNA introduced via lentiviral vectors to silence the mutation may also be used to generate cells that are amenable for use in bone regeneration (38).

Finally, while orthopaedic intervention, either by open surgery or by percutaneous delivery, are reasonable options for treatment, it would be far preferable to be able to treat patients in a more global way. Systemic injection of BMSCs has been viewed as a possible treatment of generalized skeletal disorders (42). However, there is no evidence that BMSCs delivered via the circulation are able to escape from the vasculature into extravascular spaces at a high enough concentration to have a biological impact. What can be considered is the systemic delivery of pharmacological agents, including viral vectors. The desired endpoint would be delivery of a reagent to annihilate or silence mutant BMSCs and mutant skeletal stem cells, in order to allow the normal BMSCs and normal skeletal stem cell population within the mosaic tissue to survive and to generate a bone/marrow organ.

In conclusion, FD/MAS is a disease that arises prior to gastrulation due to an accidential methylation and deamination of the CpG dinucleotide present in the R201 codon in $G_s\alpha$, resulting in R201C and R201H mutations in an embryonic stem cell. The impact of these mutations is manifested by cells of ectodermal, mesodermal and endodermal origin (café au lait and craniofacial FD; axial and appendicular FD, hyperfunctioning endocrinopathies). Post-natally, these mutations have a direct impact on the function of BMSCs and their subset of skeletal stem cells, leading to the formation of abnormal bone and a fibrotic marrow. Future therapies for the treatment of FD/MAS could be based in the manipulation of BMSC populations either by cell or molecular approaches.

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Sergei A. Kuznetsov

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Pamela Gehron Robey

I do not have any conflicts of interest.

Paolo Bianco

I do not have any conflicts of interest.

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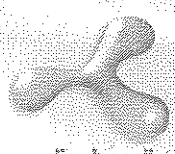

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